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In vitro regeneration of *Ensete ventricosum* from zygotic embryos of stored seeds

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Plant regeneration through *in vitro* germination and the production of adventitious shoots from zygotic embryos was investigated using stored seeds of two enset genotypes (Mariya and Oniya), as germination of intact seeds could not be achieved. The seeds were decontaminated before and after water pretreatment and the embryos cultured on MS medium. Germination was studied with vertical and horizontal embryo orientations, on three media compositions: MS medium, MS with 0.5mg l^{-1} BA + 0.2mg l^{-1} IAA and MS with 0.5mg l^{-1} BA + 0.2mg l^{-1} 2,4-D. Treating seeds of the Oniya clone with distilled water before decontamination and excision of embryos caused extensive contamination of cultured embryos. Decontamination without water pretreatment showed only about 5% contamination. Contamination was low with Mariya but no germination occurred. Horizontal embryo orientation resulted in better germination for Oniya. Supplementing MS medium with BA and IAA improved germination of the excised embryos (44%) and promoted formation of multiple shoots and simultaneous initiation of callus in some embryos. Subculturing the callus on the same medium composition (MS with BA + IAA) resulted in more and earlier initiation and proliferation of organogenic callus in the dark than other media compositions. Adventitious shoots were regenerated in the light on MS medium lacking plant growth regulators. Proliferation of a watery and friable callus was better when 2,4-D (0.2mg l^{-1}) was added in the presence of BA and IAA during subculturing.

Abbreviations: BA = benzyladenine, 2,4-D = 2,4-dichlorophenoxyacetic acid, IAA = indole-3-acetic acid, MS = Murashige and Skoog medium

Introduction

Enset (*Ensete ventricosum* (Welw.) Cheesman) is a diploid (2n = 18) herbaceous perennial belonging to the Musaceae. Enset is a staple food in Ethiopia for more than 15 million people. It produces seeds with hard seed coats. The hard seed coat of the Musaceae offers protection to the embryo during maturation, dispersal and dormancy. However, it hampers germination because the embryo requires strong forces to rupture the seed coat (Graven *et al*. 1996). These authors reported a degree of embryo-imposed dormancy in *Musa*. Intact enset seed germination is very slow and generally poor under natural conditions, as a result, seed propagation is not a common practice in enset cultivation, which is usually through vegetative suckers from corms.

Propagation by seed can, however, play an important role in enset breeding for variability and germplasm conservation. At present, enset accessions are conserved in a field genebank and are exposed to many adverse biological and physical factors. Detailed information on seed germination would be useful for germplasm conservation, as the seeds can be stored for long periods without loss of viability, complementing the field genebank. Furthermore, *in vitro* seedlings could also be good starting material for micropropagation, avoiding the difficulties in decontaminating vegetative material.

Germination of intact seeds of wild banana differed between harvest lots depending on maturity of the fruit at the time of harvest, post harvest age of the seed and method of storage (Simmonds 1952). *In vitro* culture of mature banana embryos, which were stored for three to 78 weeks after harvest, was reported (Cox *et al*. 1960). Maturity of embryos at excision and the composition of the culture medium influence germination of excised embryos (Johri and Rao 1984). Afele and De Langhe (1991) reported improved germination of excised embryos when seeds of *Musa balbisiana* were soaked in water for five days prior to embryo isolation and when the longitudinal axis of the embryo was placed flat half way embedded on the medium.

Improved germination of intact enset seeds occurred

when exposed to daily alternating temperatures (Bezuneh 1971) and when seeds were treated with hot water (Tesfaye 1992). The germination of excised embryos on simple semi-solid medium following pre-incubation of the embryos in gibberellic acid (GA_3) (Bezuneh 1980) and the use of BA and IAA-containing medium (Negash *et al.* 2000) were also reported. However, there is no information on regeneration of plants from different clones of stored enset seeds.

Plant regeneration by tissue culture techniques can be achieved by zygotic embryo culture, somatic embryogenesis, or organogenesis (Dodds and Roberts 1995). The latter approach is employed in micropropagation from bud and shoot material and in organ production from callus and suspension cultures. In the present studies, the embryos were aseptically excised from seeds of two clones of *Ensete ventricosum* that had been stored for about six years and cultured. Methods of seed decontamination prior to embryo excision, *in vitro* germination of the excised embryos (zygotic embryo culture) and regeneration of adventitious shoots from callus of the embryos (organogenesis) were investigated.

Materials and Methods

Plant material

The seeds used in these studies were obtained from Areka Agricultural Research Centre, Ethiopia. They were collected in February and March 1996 from two enset genotypes (Mariya and Oniya), sun dried and stored in brown paper bags at room temperature until used. The studies were carried out between September 2001 and August 2002.

Germination of intact seeds

Before *in vitro* culture of embryos, germination of intact seeds was examined. Intact seeds were soaked in hot water (40°C) for 30h, as described by Tesfaye (1992). Their germination was tested under three different sets of conditions: seeds were planted in pots with sand as medium; placed in petri dishes on wet filter paper; and in jars on MS medium supplemented with sucrose (30g l^{-1}) and gelled with agar (8g l^{-1}).

Germination in vitro *of embryos*

Different experiments on *in vitro* zygotic embryo germination were conducted. In all the experiments, only seeds of the two enset genotypes, that sank when placed in water, were used. The seed coat was ruptured using sterile pliers, holding seeds between thumb and forefinger. The embryos, which usually occur in the micropylar area, were removed with a scalpel and inoculated onto the medium. The basal medium of Murashige and Skoog (1962) was used with or without plant growth regulators, supplemented with sucrose (30g l^{-1}) and gelled with agar (8g l^{-1}). The medium, glassware and instruments were autoclaved at 121°C for 20min.

To compare decontamination procedures, the seeds were decontaminated after they were soaked in distilled water for 30min (water pretreatment) or without prior soaking in water (without water pretreatment). Decontamination was done for 15min in 3.5% sodium hypochlorite then rinsed three times in sterile distilled water. Embryos were then excised and inoculated onto MS medium without plant growth regulators. A 2 x 2 factorial experiment was carried out in a completely randomised design (CRD). Thirty test tubes per treatment with one embryo per test tube were used.

For *in vitro* embryo germination, the seeds were decontaminated for 15min in 3.5% sodium hypochlorite, then rinsed three times in sterile distilled water. Factorial combinations of two types of embryo orientations on the three different medium compositions were used in a CRD. Embryo orientations on the medium were vertical (haustorium embedded in medium with the meristematic region exposed) or horizontal (longitudinal axis of the embryo was placed flat halfway embedded on medium). The composition of the media were: (1) MS without plant growth regulators (PGRs) and (2) MS supplemented with (in mg l^{-1}) 0.5 BA + 0.2 IAA or 0.5 BA + 0.2 2,4-D. After inoculation, the cultured embryos were transferred to a growth room and incubated in the dark at 24°C. Three weeks later, primary roots and the hypocotyl-epicotyl parts of the embryo emerged. Seedlings were then transferred to irradiances of 4–6µmol m^{-2} s^{-1} for a week and thereafter to 43µmol m^{-2} s^{-1}.

Callus proliferation and plant regeneration

Callus was obtained from the embryos cultured on the medium containing BA + IAA. The callus was multiplied on the same medium composition, adding 1gl^{-1} casein hydrolysate as a source of organic nitrogen. Proliferation of the callus and plant regeneration were studied on MS medium with five types/concentrations of PGRs. These were (mgl^{-1}): 0.5 BA + 0.2 IAA (T1); 0.5 BA + 0.2 IAA + 0.1 2,4-D (T2); 0.5 BA + 0.2 IAA + 0.2 2,4-D (T3); 1 BA + 0.4 IAA + 0.4 2,4-D (T4); and 1.25 2,4-D (T5). Callus was maintained and multiplied by subculturing every eight weeks onto T2 + 1gl^{-1} casein hydrolysate for eight months after callus initiation. Thereafter, the callus was treated on MS with different PGRs and concentrations (mgl^{-1}): 1.13 BA (A_0C_1); 4.51 BA (A_0C_2); 0.28 2,4-D + 1.13 BA (A_1C_1); and 0.28 2,4-D + 4.51 BA (A_1C_2). Shoots were regenerated from organogenic callus on MS medium without PGRs in the light, using a 16/8h light/dark cycle and an irradiance of 43µmol m^{-2} s^{-1} at 24°C.

Data collection and statistical analysis

Data on germination and contamination, number of shoots per embryo, shoot height, root length and number of roots and leaves were collected. Mean percent of organogenic, watery and friable callus was computed from values from 10 petri dishes per treatment. Adventitious shoots with each treatment were counted. GenStat 5 release 4.1 was used to analyse the data. Significant means were separated by least significant differences (LSD) at a 5% probability. Standard errors of means (SE) were also computed. Correlation matrix was run to explain associations of growth parameters.

Results and Discussion

Germination of intact seeds

Hot water treatment of intact seeds did not result in germination. However, by rupturing the seed coat physically, two months after application of the hot water treatment, elongation of embryos was observed in some seeds of the Oniya clone placed on wet filter paper in petri dishes or on sucrose medium in jars. This suggested that the elongating radicle-epicotyl parts of the embryos could not push through the micropylar collar of the seed coat and as a result the embryos died. Stotzky *et al*. (1962) reported a condition where embryos could not emerge through the micropylar canal. As the chalazal mass was removed in their study the embryo emerged from the bottom of the seed rather than through the micropylar canal. There was a slight dark blue coloration of the medium indicating release of seed components. Remarkable things about the seed coat structure of the Musaceae are the micropylar collar, the relatively thick mesotesta, the unique macromolecular composition with typical Musaceae phenolic compounds and the breaking of the cell walls in the exotestal layer resulting in a surface with silica crystals (Graven *et al*. 1996). According to McGahan (1961), the seeds of *Ensete* differ from those of *Musa balbisiana* by being considerably larger and by having a conspicuous hilum cavity; otherwise the differences appear to be ones of degree rather than kind.

Bezuneh (1980) described the basic morphology of the enset embryo which has similarities with the embryo of *Musa* (McGahan 1961). The embryo also has the mushroom shape, characteristic of the Musaceae. The enlarged cap like portion, the haustorium, is the principal part of the cotyledon while the stalk-like portion, as well as a part of the cotyledon (McGahan 1961) represents the epicotyl-hypocotyl-radicle axis. The stalk-like portion is located within the micropylar collar.

Decontamination of seeds and in vitro *germination of embryos*

Seeds were first pretreated with water for four days prior to decontamination to test the effect of imbibition of water on embryo germination. However, because of extensive contamination of the embryos germination could not be observed. Seeds were then pretreated with water for only 30min prior to decontamination to determine if the water pretreatment really caused the contamination. Different levels of contamination of cultured embryos were observed depending on enset genotype and water pretreatment before decontamination (Figure 1). Embryos from seeds of Oniya pretreated with water and then decontaminated were highly contaminated (95%) while embryos from the same genotype and seed lot, decontaminated without prior water treatment, gave 5% contamination. This was the only treatment where embryo germination occurred (18%). While the embryos of Mariya were only slightly contaminated irrespective of treatment no germination occurred.

The seeds of the Mariya clone were clean around the hilum and the micropylar plug was exposed. In some seeds the plugs (seed lids) were dislodged, possibly during seed harvesting and storage. In the case of Oniya seeds the hilum was covered with dry tissues and the micropylar plugs were not exposed. From the results it seems that exposing and/or removing the seed plug had an adverse effect on embryo viability during storage. While dead tissue around the hilum harboured contaminants the seeds remained viable.

The interaction effects between genotype and orientation of embryos and between genotype and medium composition influenced *in vitro* germination of the embryos (Table 2). This would be because the embryos from the seeds of the Mariya clone failed to germinate under all treatment combinations. For Oniya, orienting the embryos horizontally on the medium resulted in a higher germination percentage than when placed vertically (Table 1). Horizontal placement of the embryos which increased germination of enset embryos also improved *in vitro* germination of *Musa balbisiana* embryos (Afele and De Langhe 1991). Exposing part of the haustorium appeared to favour embryo germination.

In general, mature embryos require only inorganic salts supplemented with sucrose, whereas immature embryos have an additional requirement for vitamins, amino acids, growth regulators and sometimes coconut milk or some other endosperm extract (George and Sherrington 1984, Hu and Wang 1986). However, MS basal medium supplemented with BA and IAA resulted in higher germination of enset embryos than MS medium without PGRs and with BA + 2,4-D. Use of BA alone did not significantly improve germination of *Musa acuminata* embryos (Asif *et al*. 2001).

Effect of medium composition on multiple shooting and shoot growth

The number of shoots per embryo, formation of multiple shoots per treatment, and vegetative growth of the seedlings (shoot height, number of leaves per shoot and length of root) were significantly influenced by medium composition (Table 3). Shorter shoots and roots and fewer leaves were recorded in the presence of BA + IAA, with on average 37% of the

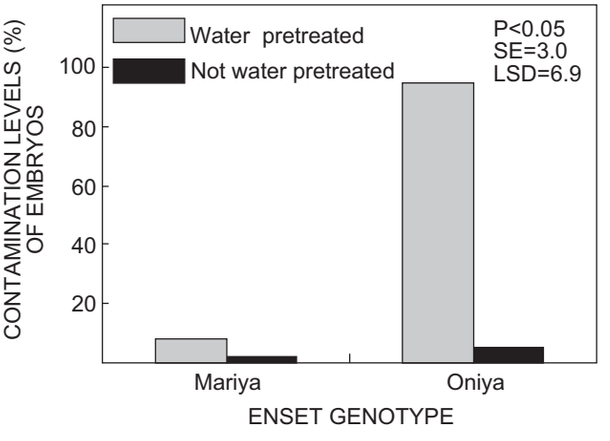

Figure 1: Contamination levels (%) of zygotic embryos cultured *in vitro* as influenced by water pretreatment of seeds for 30min before seed decontamination for the two enset genotypes, Mariya and Oniya

Table 1: Effect of embryo orientation, medium composition and enset genotype on *in vitro* germination (%) of the embryos

Treatments	Enset genotype	
	Mariya	Oniya
Embryo orientation		
Vertical	0	29
Horizontal	0	38
SE	0.5	
LSD (5%)	1.4	
Medium		
MS	0	25
MS + BA + IAA	0	44
MS + BA + 2,4-D	0	32
SE	0.6	
LSD (5%)	1.8	

Table 2: Statistical significance of treatment effects (F-test) on *in vitro* embryo germination of the two enset genotypes, Mariya and Oniya

Treatment	F-probability
Enset genotype	<0.001*
Embryo orientation	<0.001*
Medium	<0.001*
Genotype x orientation	<0.001*
Genotype x medium	<0.001*
Orientation x medium	<0.599ns
Genotype x orientation x medium	<0.599ns

* = significant
ns = non-significant

Table 3: Growth of shoots from embryos of Oniya enset genotype as affected by *in vitro* treatments indicated by values of F-probabilities

Medium composition	No. of shoots embryo^{-1}	Multiple shoot formation	Shoot height (cm)	No. of leaves shoot^{-1}	No. of roots embryo^{-1}	Length of root (cm)
Medium (M)	0.010*	0.002*	0.019*	0.010*	0.208	0.021*
Orientation (O)	0.121	0.056	0.231	0.710	0.157	0.435
M x O	0.055	0.230	0.581	0.593	0.375	0.701

* Significant at a 5% probability

Table 4: Effect of medium composition on *in vitro* growth of shoots from embryos of Oniya genotype, three months after embryo culture

Medium composition	No. of shoots embryo^{-1}	Multiple shoot (%)	Shoot height (cm)	No. of leaves shoot^{-1}	No. of roots embryo^{-1}	Length of root (cm)
MS	1.7	15.0	3.1	3.2	2.7	1.6
MS + BA + IAA	4.5	37.0	1.8	2.2	2.7	0.5
MS + BA + 2,4-D	1.0	0.0	7.7	4.7	4.2	4.0
SE	0.6	4.0	1.1	0.4	0.8	0.6
LSD (5%)	1.9	13.9	3.7	1.3	ns	2.2

embryos producing multiple shoots (Table 4). Multiple shoots were also formed when medium without PGRs was used. The maximum number of shoots per embryo was three (average 1.7) from MS medium and eight (average 4.5) from BA + IAA containing medium. It was reported that MS medium with BA + IAA resulted in clumping of shoots (Negash *et al*. 2000) in germination of enset zygotic embryos.

The correlation matrix in Table 5 shows an inverse relationship between formation of multiple shoots and growth of the seedlings. As the number of shoots per embryo increased, shorter plants and roots and fewer leaves per shoot were obtained. Taller shoots produced more leaves and roots than the shorter ones. Formation of the multiple shoots resulted in reduced sizes of shoots. However, about 90% of the multiple shoots have grown into complete seedlings after separating and subculturing them individually. Inclusion of activated charcoal into the medium improved growth of the shoots.

Table 5: Associations of number of shoots per embryo and other shoot growth parameters for Oniya enset genotype

NS/E	1.000				
SH	–0.652*	1.000			
NL/S	–0.616*	0.666**	1.000		
NR/E	–0.227	0.823**	0.348	1.000	
RL	–0.565*	0.699**	0.785**	0.322	1.000
	NS/E	PH	NL/S	NR/E	RL

NS/E = Number of shoots per embryo
SH = Shoot height
NL/S = Number of leaves per shoot
NR/E = Number of roots per embryo
RL = Root length (average) (n = 24)
* and ** indicate significant correlations between growth parameters at 5% and 1% probabilities, respectively

Table 6: Effect of medium composition on growth of callus and regeneration of adventitious shoots from zygotic embryos of Oniya enset genotype, five months after callus initiation. Treatments (mgl^{-1}): T1 = 0.5 BA + 0.2 IAA, T2 = 0.5 BA + 0.2 IAA + 0.1 2,4-D, T3 = 0.5 BA + 0.2 IAA + 0.2 2,4-D, T4 = 1 BA + 0.4 IAA + 0.4 2,4-D and T5 = 1.25 2,4-D

Treatments	Type of callus (%)			No. of adventitious shoots jar^{-1}
	Watery and friable	Organogenic	Brown	
T1	24	76	0	5
T2	47	52	0	5
T3	58	42	0	5
T4	32	24	43	4
T5	0	0	93	0
SE	2	1	1	1
LSD (5%)	6	6	4	2

Table 7: Regeneration of adventitious shoots of Oniya enset genotype from 8-month-old callus (data after four months on regeneration medium (MS)). Concentrations of PGRs ($mg1^{-1}$): A_0C_1 = 1.13 BA, A_0C_2 = 4.51 BA, A_1C_1 = 0.28 2,4-D + 1.13 BA and A_1C_2 = 0.28 2,4-D + 4.51 BA

Type of media		Number of shoots jar^{-1}		Longest shoot (cm)	Number of leaves
For callus	For shoot regeneration	Healthy	Vitrified		
A_0C_1	MS	2	0	8	3
A_0C_2	MS	2	4	5	3
A_1C1	MS	6	10	2	2
A_1C_2	MS	4	4	3	2

Regeneration of adventitious shoots from callus

Callus was only obtained from embryos cultured on MS medium supplemented with BA + IAA. Nevertheless, on BA + 2,4-D-containing medium enhanced swelling of the epicotyl-hypocotyl portion of germinating embryos was observed. The swelling produced layers of thickened leaf sheaths on the seedlings, being abnormal. Upon transferring the seedlings to MS medium without PGRs the first leaf sheaths produced became brown and were replaced by new normal leaf sheaths.

The effects of medium composition on growth of callus and regeneration of adventitious shoots are given in Table 6. Callus developed into heterogeneous structures: watery, friable and organogenic callus; even within the same treatment. However, there were clear treatment effects. T1 resulted in a large amount of organogenic callus and earlier shoot growth while T3 followed by T2 was good for production of watery and friable callus. Increasing BA, IAA and 2,4-D from 0.5mg l^{-1}, 0.2mg l^{-1} and 0.2mg l^{-1} to 1mg l^{-1}, 0.4mg l^{-1} and 0.4mg l^{-1}, respectively, did not improve proliferation of callus. T5 (1.25mg l^{-1} 2,4-D) resulted in browning and inhibited growth of callus. Regeneration of the adventitious shoots was also achieved on A_0C_1, A_0C_2, A_1C_1 and A_1C_2 (Table 7) media from callus maintained for eight months. The most important characteristic of callus, as stated by Dodds and Roberts (1995), in general, is that it has a potential to develop normal roots, shoots and embryoids that can form plants. In these experiments, vigorous adventitious shoots with roots were regenerated from organogenic callus. Regeneration of roots occurred following growth and development of shoots. Zeweldu (1997) also reported regeneration of roots as well as shoots from callus of shoot tips grown on a medium with 0.018mg l^{-1} IAA + 0.225mg l^{-1} BA. The adventitious shoots can be used as plant material or breeding material based on genetic uniformity.

From the present results it can be concluded that, based on various factors, enset seeds remain viable for a long time, six years in this case, and normal plants can be regenerated from them.

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